Engineered cytochrome P450 BM3 mutants as biocatalysts for metabolite production: Applications in biotechnology and toxicology

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2014

Chapter 8

SUMMARY, CONCLUSIONS AND PERSPECTIVES

Summary

The application of enzymes as biocatalysts in chemical and pharmaceutical industry has gained considerable importance over the years. Enzymes are capable of catalyzing a wide range of reactions with high selectivity under mild conditions. Oxidative biocatalysts are especially of great significance because of the ease with which they are capable of catalyzing many reactions including the selective oxidation of unactivated C-H bond (*1, 2*). Regio- and stereoselective oxidation of compounds ranging from fine chemicals to drug like compounds is of high value in the chemical industry. This thesis covers the potential of cytochrome P450 BM3 mutants for metabolite production for different applications.

The main goal of this thesis was to engineer bacterial cytochrome P450 BM3 enzyme for regio- and stereoselective hydroxylation of fine chemicals, steroids and drugs. Novel site-directed mutants specific for different applications have been developed. Mutants M01 and M11 which were generated prior to this study (*3*), were used as templates for additional site-directed mutagenesis of active site residues guided by computational modelling of the active site of P450 BM3. The engineered P450 BM3 mutants mimicking human liver mcirosomes were used for the synthesis of drug metabolites for toxicological studies. Additionally, engineered P450 BM3 mutants were used for the large scale production of GSH conjugates to enable structural elucidation by NMR and for bioactivation of drugs to study the effect of human glutathione-S-transferases.

The work described in chapters **2, 3 and 4** of this thesis was performed as a part of an IBOS project titled "*Biocatalytic exploitation of monooxygenases*" (**BIOMOX**). The aim of this part was to engineer and characterize P450 BM3 enzymes for selective oxidation of fine chemicals and steroids. The last 3 chapters (**5, 6 and 7**) describe the use of engineered cytochrome P450 BM3 variants for drug metabolite production and their potential use in toxicological studies.

Chapter 1 gives a general introduction to the various scientific aspects covered in this thesis. In this chapter, the potential of oxidative biocatalysts and in particular monooxygenases is discussed. Cytochrome P450s are a class of heme-dependent mono-oxygenases which metabolize more than 75 % of the drugs in the market. Although human P450s are widely known for their biotransformation of xenobiotic compounds, their use at preparative scale is limited because of their low stability and membrane bound nature. Microbial P450s are soluble and easier to express and therefore are an attractive alternative for metabolite synthesis compared to their

human counterparts (*4*). Protein engineering of bacterial P450s and specifically P450 BM3 has proved to be an excellent means to broaden their substrate scope, specificity and to improve their activity. Recent examples of protein engineering from literature to illustrate the tuning of bacterial P450s for selective oxidations are discussed in this chapter (*5, 6*).

The preparative scale synthesis and isolation of drug metabolites is of utmost importance in light of the recent regulatory guidelines of FDA (*7*). There is a growing need to find green alternatives for synthesis of drug metabolites (*8*). The optimization of P450 BM3 for improved production of drug metabolites is also discussed in this chapter. Apart from the metabolism of drugs, P450s are also involved in bioactivation reactions leading to the formation of toxic reactive intermediates. These reactive metabolites can lead to covalent modifications of proteins thus resulting in adverse drug reactions. Development of effective methods for detection and structural elucidation of reactive metabolites early in drug development is important for the development of safer drugs (*9*). Different strategies employed for reactive metabolite and glutathione (GSH) conjugates generation are discussed in this chapter. The conjugation of a reactive metabolite to GSH can be spontaneous or catalyzed by human glutathione-S-transferases (hGSTs) (*10*). The role of hGSTs in the detoxification of the reactive metabolites by catalyzing GSH conjugation reactions is also covered in this chapter.

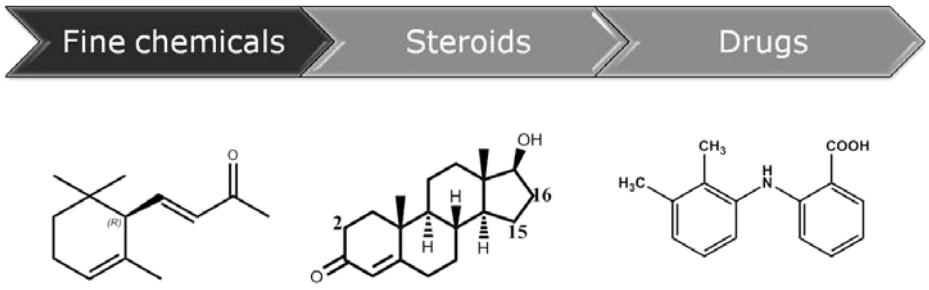

Figure 1. Examples of compounds used in this thesis as substrates for engineered P450 BM3 variants

Enzymes capable of selective oxidation of fine chemicals are of high value in the chemical industry (*11*). α-ionone and its hydroxylated variants have organoleptic properties and are of commercial interest in the flavour and fragrance industry. In **chapter 2**, a library of P450 BM3 mutants was screened for their potential to selectively hydroxylate α-ionone. Initial screening with racemic α-ionone revealed that mutants M01 A82W, M11 A82W and M11 V87I selectively hydroxylated α-ionone

at C-3 with *trans* stereoselectivity while mutants of M11 with mutation at 437 position formed almost equal amounts of both *cis* and *trans* diastereomers of 3-OH-α-ionone. Interestingly, incubation with individual enantiomers (6*R*)- and (6*S*)-α-ionone showed that the mutant M11 L437N showed opposite stereoselectivity resulting in the formation of (*3S,6R)-OH* and (*3S,6S)-OH*-α-ionone respectively with >90% *d.e*. One of the mutants tested M11 V87F L437N showed moderate selectivity (40%) for the formation of (3*R*, 6*S*)-OH diastereomer which could be used as a starting template for further optimization.Docking studies identified the importance of residue at position 82 in the stereoselective hydroxylation of α-ionone enantiomers. These engineered P450 BM3 mutants with high total turnover numbers can be used for preparative scale oxidation of small molecules like α-ionone.

In **chapter 3**, the potential of *Rhodococcus erythropolis* as a host for P450 BM3 mediated whole cell biocatalysis was explored using norandrostenedione (nordione) as a model substrate. The wildtype *Rhodococcus* strain breaks down steroids because of its 9α-hydroxylase and 3-ketosteroid dehydrogenase activities, thus hampering its use as a host for steroid bioconversion. By distrupting the genes encoding these catabolic enzymes, several mutants of *Rhodococcus erythropolis* do not degrade steroids. One such mutant RG9 was used in this chapter to heterologously express P450 BM3 mutant M02 and study the biotransformation of nordione. P450 BM3 variant M02 has been shown to convert steroids with high selectivity and therefore was chosen for this study. Using *in vitro* biotransformation, the structure of the product was first determined by NMR to be 16-β-OH-nordione. The BM3 variant M02 was then heterologously expressed in RG9 and the biotransformation was studied. About 0.35 g/L of the product was formed which corresponded to the product 16-β-OH-nordione formed *in vitro*. Therefore, the product conversion in RG9 is P450 BM3 mediated. This study shows that the combination of mutant *Rhodococcus* strain and a highly active engineered P450 BM3 variant is a promising option for whole cell biocatalysis.

In **chapter 4**, a semi-rational approach was used to identify the key active site residues involved in the binding of testosterone in P450 BM3. Residues 72, 82, 87 and 437 were identified to have an effect on the binding orientation of testosterone. A minimal library consisting of combinatorial single and double mutations at these residues was constructed. At the time of this study, P450 BM3 mutants capable of hydroxylation of only A or D ring on the β-face of testosterone were reported. Screening of the minimal library revealed that two mutants were able to produce a new metabolite which was not reported before. One of these BM3 mutants, M01 A82W S72I was to used to scale up the reaction and the new metabolite was identified

by a combination of HPLC co-elution, GC-MS and NMR analysis to be 16-α-hydroxy-testosterone. The introduction of S72I mutation led to about four fold decrease in the catalytic activity in comparison to its parent M01 A82W. Nevertheless, this mutant could invert the stereoselectivity of testosterone C-16 hydroxylation by only one mutation. The parent mutant M01 A82W was known to catalyze C-16 hydroxylation with 100% β-enantioselectivity. Introduction of this mutation in another 16-β-hydroxylase variant M11 V87I also resulted in a similar change in enantioselectivity. *In-silico* modelling studies provided insights into active site interactions responsible for this change in selectivity. By replacing the polar serine with a hydrophobic isoleucine at position 72, the hydrogen bond formation with the A-ring carbonyl group of testosterone which was necessary for 16-β hydroxylation was removed. In addition, different rotamers of isoleucine showed steric hindrance of its side chain with testosterone bound in a conformation suitable for 16-β hydroxylation. This study showed that small differences in the key substrate interaction residues in P450 BM3 can have a profound effect on the stereoselectivity of hydroxylation.

In **chapter 5**, the applicability of P450 BM3 mutants for the generation of human relevant metabolites of fenamic acid NSAIDs was studied. A library of mutants was screened with meclofenamic acid to identify catalytically active and selective mutants. Mutants of M11 with isoleucine and phenylalanine at position 87 were found to selectively hydroxylate at benzylic and aromatic positions of meclofenamic acid. Engineered P450 BM3 variants were used as biocatalysts to enable structural elucidation of metabolites by NMR. The isolated metabolites were found to be 3'-hydroxy-methyl, 4'-hydroxy and 5-hydroxy-meclofenamic acid. These mutants were also found to hydroxylate structurally similar compounds mefenamic acid and tolfenamic acid with high selectivity and total turnover numbers ranging from 4000-6000. It was found that while both mefenamic acid and meclofenamic could undergo benzylic as well as aromatic hydroxylation by engineered P450 BM3 variants, tolfenamic acid was hydroxylated only on aromatic rings. Computational studies helped to rationalize the observed aromatic ring selectivity of P450 BM3 mediated hydroxylation. The enzymatically synthesized metabolites can be used as references for cellular studies as well as for further toxicological evaluation.

In **chapter 6**, engineered P450 BM3 mutants and human liver microsomes were used for the *in vitro* bioactivation of drugs like clozapine, acetaminophen and diclofenac to their corresponding reactive metabolites in order to study the ability of four allelic variants of hGSTP1-1, namely hGSTP1*A (Ile105/Ala114), hGSTP1*B (Val105/Ala114), hGSTP1*C (Val105/Val114) and hGSTP1*D (Ile105/Val114), to catalyze the GSH conjugation of the reactive metabolites. Differences in activity

between the proteins could not be attributed to a general decrease in catalytic efficiency. Rather, the differences reflected the effect of residue 105 and 114 specific for any given substrate. Single substitutions at residue 105 or 114 did affect the ability to catalyze GSH conjugation. However, when both residue 105 and 114 were substituted the effect could be enhanced or diminished. Based on the results in this chapter, we suggest that the binding orientation of substrates in the active site of GSTP1 mutants is changed and has effect on GSH conjugation.

Chapter 7 describes the use of cytochrome P450 BM3 as a tool to bioactivate mefenamic acid (MFA) to reactive benzoquinoneimine intermediates and for the structural elucidation of the GSH conjugates. The high activity of BM3 mutant M11 resulted in the convenient large scale production and isolation of the major GSH conjugate. The structure of the major conjugate was elucidated by NMR analysis and was found to be derived from MFA-1',4'- quinoneimine followed by subsequent GSH conjugation at 6' position. Two more minor conjugates were found to be derived from the MFA-2,5-quinoneimine pathway. MFA was incubated with fourteen different recombinant human CYPs to elucidate enzymes responsible for its bioactivation. While P450 mediated benzylic hydroxylation was catalyzed by CYP 2C9, the aromatic hydroxylation leading to the formation of 1',4'-quinoneimine and the major GSH conjugate was catalyzed by both CYP 1A2 and CYP2C9. The formation of 5-OH-MFA and the minor GSH conjugates was mainly catalyzed by CYP1A2. Inhibition of metabolite formation by addition of selective inhibitors of individual CYP enzymes to human liver microsomes incubations confirmed these results. Since P450 BM3 could bioactivate MFA to the same human relevant intermediates, it was used as a tool to study the bioactivation and subsequent inactivation of the reactive quinoneimines by glutathione-S-transferases. The effects of a panel of seven major recombinant human GSTs on the formation of GSH conjugates of MFA were studied. The formation of the major GSH conjugate was not significantly increased by the addition of hGSTs. However, the formation of minor conjugates was catalyzed by hGSTs, in particular the addition of hGST P1-1 led to a ten-fold increase in the formation of one of the regioisomeric conjugates derived from 5-OH pathway.

Conclusions

During the last ten years, several labs around the world have worked on developing novel mutants of cytochrome P450 BM3 for a variety of applications (*6*). In the beginning, focus was laid on selective oxidation of small molecules and aromatics which over the years has shifted to exploit the catalytic potential of P450 BM3 enzymes for pharmaceutical applications (*12*) and novel reactions like

cyclopropanation and C-H amination not known thus far for P450s (*13, 14*). **Figure 2** summarizes the powerful tool of protein engineering used in context of P450 BM3 and the various novel applications of the engineered P450 BM3 variants.

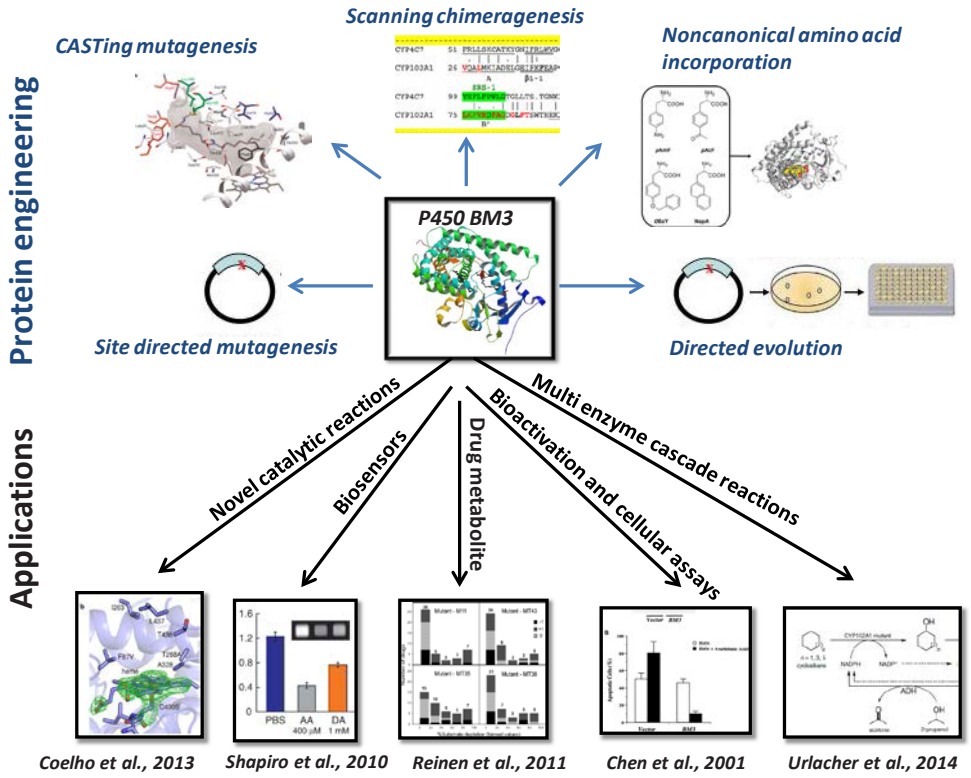

Figure 2. The figure shows protein engineering approach (top panel), and applications of engineered P450 BM3 mutants (bottom panel). Pictures depicting the various approaches to engineer P450s are taken from references (*15-18*). Pictures depicting the applications of the engineered variants were reproduced from references (*13, 19-22*). The general template of this figure is adapted from (*18*).

Future prospectives based on this thesis

Some aspects described in this thesis that be applied in future studies are discussed below.

Chimeragenesis as an approach for B or C ring steroid hydroxylation

The regio- and stereoselectivity of P450-mediated reactions depends upon the orientation of the substrate relative to the reactive iron-oxo species, which is in turn determined by the active-site configuration of the P450 enzyme. (*23*). P450 BM3 mutants engineered by combinatorial saturation mutagenesis or alanine scanning mutagenesis can perform only A or D ring hydroxylation of steroids (*16, 24*). It still remains a challenge to achieve B or C ring hydroxylation such as 6β-hydroxy-testosterone which is the major human metabolite of testosterone. These observations reiterate the fact that more rigorous methods may be required to re-shape the active site to enable B or C ring hydroxylation. One approach is to create chimera of cytochrome P450s by domain swapping or by scanning chimeragenesis which has been successfully employed to for other substrates (*15, 25*). For example, in a recent study, the substrate recognition sites of insect CYP4C7, which hydroxylates farnesol at the terminal C12 position with high selectivity but low activity, were introduced into P450BM3 to yield successful chimeras capable of producing 12-hydroxy-farnesol not observed with wild-type P450BM3(*15*).

Computational and structural studies

In **chapters 2 and 4**, we show that *in silico* docking studies can provide insights into the structural rationalization of the experimentally observed regio- and stereoselective hydroxylation of α-ionone and testosterone. In these studies, computational methods were used to identify critical residues involved in substrate recognition and to rationalize the experimentally observed selectivity. More recently, free energy calculations were applied to study the stereoselective hydroxylation of α-ionone enantiomers by engineered BM3 mutants (*26*). In **chapter 5** of this thesis, the regioselective benzylic and aromatic hydroxylation of fenamic acid NSAIDs is described. *In silico* modelling studies were used to get preliminary insights into the ring preference of fenamic acid hydroxylation by P450 BM3 mutants. In future studies, the observed benzylic and aromatic hydroxylation selectivity in different mutants can be further rationalized in extensive molecular dynamics (MD) simulation based studies by including protein plasticity and induced fit effects. This will be in line with the previous studies in which it was shown that predicting the effect of P450 BM3 small active site mutations on regio-or stereoselectivity of substrates was found to be difficult based on (rigid) docking and scoring only (*27*). Moreover, crystallization of the selective P450 BM3 variants in the ligand bound and ligand free forms can potentially help to unravel the conformational changes caused by the mutation and get insights into the structural basis of selective oxidation.

Engineered P450 BM3 as a bioactivation tool in mammalian cellular studies

The detection of the possible bioactivation pathways of drugs to reactive metabolites in early stage drug discovery is often difficult because of the problem of characterizing low levels of reactive metabolites. Therefore, efficient methods for the generation and characterization of reactive metabolites are necessary (*28*). In **chapter 6 and 7**, P450 BM3 mutants are used as biocatalytic tools to generate high amounts of reactive intermediates. Recently, *Boerma et.al* used engineered P450 BM3 mutants to generate protein adducts resulting from the bioactivation of many drugs (*29*). Moreover, engineered P450 BM3 mutant F87V which metabolized arachidonic acid only to *14,15*-epoxyeicosatrienoic acid (14,15-EET) was transfected in pig renal epithelial cell lines (LLCPKcl4) as a tool to study the role of arachidonic acid epoxygenase pathway. The stable transfectants of of LLCPKcl4 cells with P450 BM3 mutant F87V as well as empty vector-transfected LLCPKcl4 cells were used to investigate the cell survival effects of endogenously produced 14,15-EET (*20, 30*). These studies exemplify the diverse applicability of engineered P450 BM3 which can be extended to mammalian cellular toxicity models in future.

To summarize, engineered P450 BM3 mutants have been shown to have the potential as biocatalysts for metabolite production with possible applications ranging from biotechnology to toxicology. Combinatorial active site mutations have been effective to improve the selectivity of the reactions as well as to understand the structure-function relationship of P450 BM3 reflected by the synergy between the experimental and computational studies. Moreover, the combination of P450 BM3 variants with other enzymes has led the development of a potential study system that can be applied for the large scale generation of reactive metabolites for structural elucidation as well as a versatile tool to study the interplay between Phase I and Phase II enzymes.

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